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Relationship between HLA class I antigen processing machinery component expression and the clinicopathologic characteristics of bladder carcinomas

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Abstract

Purpose—The goal of this study was to analyze protein expression of antigen processing machinery (APM) components in bladder carcinoma (BC), and to assess the clinical significance of defects in their expression.

Experimental design—Tissue from 167 cystectomies for primary BC was used to create a tissue microarray. 128 tumors were urothelial carcinoma (UC). Immunohistochemistry was performed using 14 monoclonal antibodies to APM components (β 2-microglobulin, calnexin, calreticulin, delta, Z, MB1, LMP2, LMP7, LMP10, HLA class I heavy chain, tapasin, TAP1, TAP2 and ERp57) and MHC class I-related antigen (MICA). Sections of normal urothelium from six subjects were used as controls.

Results—All APM components except MB1, LMP2 and TAP2 had significantly lower staining in UC than in normal urothelium. No significant differences were found in APM component scores between different grades of UC. Squamous cell carcinoma had the highest scores, with UC intermediate and other types of BC lowest. High-stage UC demonstrated significantly lower

staining for calnexin, LMP2, LMP7 and LMP10 than low-stage UC. With mean 3.6 years follow up, significantly worse survival was associated with a higher delta score in UC ($P = 0.038$) and a lower calreticulin score in all tumor types ($P = 0.028$).

Conclusions—Most APM components were downregulated in UC. High-stage UC had lower scores for immunoproteasome components compared to low-stage UC. Delta and calreticulin protein expression was associated with survival in UC and in all types of BC, respectively. These findings suggest that APM defects play a role in the clinical course of BC and should be considered in developing immunotherapeutic approaches for its control.

Keywords

Antigen processing machinery; Bladder; Immune escape; HLA class I; Transitional cell carcinoma; Urothelial carcinoma

Introduction

Sixty-one thousand new cases and 3,000 deaths from bladder cancer were predicted in the United States in 2006 [1]. Although the relative 5-year survival rate for muscle invasive bladder cancer in the United States has improved over the last 40 years, patients who develop metastatic disease following definitive local therapy are only rarely curable. Two large studies of patients having radical cystectomy found 10-year overall recurrence-free survival rates of 66 and 50% with and without neoadjuvant therapy, respectively [2, 3].

The growth of many carcinomas including those of the bladder may be partially controlled by cytolytic T-lymphocytes (CTL), whose T-cell receptors (TCR) recognize antigens bound to major histocompatibility (MHC) class I molecules on the surface of tumor cells [4]. Tumor cells may avoid detection and destruction by the immune system either by losing or down-regulating tumor-associated antigens and/or antigen processing machinery (APM) components.

Several steps lead to the generation of tumor antigen-derived peptides which are presented by HLA class I antigens to cognate CTL. Specifically, tumor antigens are degraded by the proteasome (delta, β 2 and MB1) and the immunoproteasome [low-molecular weight protein (LMP) 2, LMP7 and LMP10]; the latter is induced by the action of inflammatory cytokines such as interferon- γ and TNF- α [5]. The antigens are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), which is composed of the two noncovalently associated subunits, TAP1 and TAP2. The ER chaperones calnexin, calreticulin, and the thiol oxidoreductase ERp57 allow for the correct folding of the HLA class I- β 2 microglobulin (β 2m) complex, which then associates with tapasin and subsequently with TAP. This facilitates peptide loading onto the HLA class I- β 2m complex, after which the antigenic peptides may be trimmed by an ER-associated aminopeptidase. The HLA class I- β 2m-peptide complex then travels to the plasma membrane via the Golgi apparatus. MHC class I-related antigen or MICA plays no role in antigen presentation, but acts as a ligand for a receptor on natural killer cells, CD8+ T cells and $\gamma\delta$ T cells [6]. The MICA protein is expressed in several endothelial and hematopoietic tumor types and activates effector functions of NK cells in mucosal tissues.

Levels of APM components have been studied in several types of human carcinomas and in malignant melanoma, but have not been thoroughly examined in bladder carcinomas (BC). This reflects at least in part the lack of APM component-specific antibodies which stain formalin-fixed, paraffin-embedded tissues. Monoclonal antibodies (mAb) with these characteristics have recently been developed by one of us (SF). Using these reagents, we have interrogated a large group of BC for their levels of APM components and compared the

results with the histopathologic type of carcinoma, tumor grade and pathologic stage. In addition, we have examined the relationship between APM protein expression and patient survival.

Materials and methods

Bladder carcinomas and normal urothelium

This study was approved by the University of Virginia Institutional Review Board for Health Sciences Research. All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 Declaration of Helsinki. The files of the Department of Pathology at the University of Virginia were searched for patients with BC initially diagnosed from 1990 to 2001, and treated with cystectomy. All tumors were reviewed to confirm the diagnosis of BC. Cases with minimal residual tumor were excluded. A tissue microarray containing four 0.6-mm cores obtained from 167 cystectomies for primary carcinoma of the bladder was constructed from zinc formalin-fixed, paraffin-embedded blocks (Beecher Instruments, Silver Spring, MD). Slides from the resections were reviewed for histologic subtype, grade (1–4) of urothelial carcinoma (UC), and pathologic stage [7]. Overall survival information was obtained from medical records and from the files of the McIntire Tumor Registry at the University of Virginia. Disease-specific survival data were not available.

Histologically normal-appearing urothelium from two cystectomy specimens and four ureters taken from resections for genitourinary cancer were used as controls. Normal epithelium from patients with tumor in this study was not examined.

Mono- and polyclonal antibodies

Z-specific mAb NB1 [8]; delta-specific mAb SY-5 [8]; MB-1-specific mAb SJJ-3 [8]; LMP2-specific mAb SY-1 [9]; LMP7-specific mAb HB2 [9]; LMP10-specific mAb TO-7 [9]; TAP1-specific mAb NOB-1 [8]; TAP2-specific mAb NOB-2 [8]; calnexin-specific mAb TO-5 [10]; calreticulin-specific mAb TO-11 [10]; ERp57-specific mAb TO-2 [10]; tapasin-specific mAb TO-3 [10]; mAb HC-10 which recognizes a determinant expressed on β 2m-free HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains and on all β 2m-free HLA-B and C heavy chains [9, 11]; β 2m-specific mAb L368 [12, 13]; and MICA-specific mAbs WW9B8 and WJ-1, (from the laboratory of SF [14]), were developed and characterized as previously described. Biotinylated anti-mouse IgG xenoantibodies were purchased from Vector (Burlingame, CA).

Immunohistochemical staining of the tissue microarray

Deparaffinized, zinc formalin-fixed, paraffin-embedded tissue sections were placed in citrate buffer pH 6.0 and heated in a microwave oven for 10 min. Following a 60-min incubation with an optimal dilution of primary antibody at room temperature, slides were incubated with an optimal amount of the biotinylated secondary antibody for 20 min at room temperature, then avidin–biotin immunoperoxidase was applied. Diaminobenzidine was used as the chromogen, and sections were counterstained with hematoxylin. Staining was scored by multiplying the percent of stained tumor cells (0; 1: 1–25% positive; 2: 26–75%; 3: 76–100%), by the staining intensity (1: weak; 2: strong). The protein expression of APM components was classified as low, when the score was 3 or less, and high when the score was >3. Specifically, the expression of an APM component was classified as high, when the percent of tumor cells was >25% and the staining intensity was strong, and as low whenever the staining intensity was weak. Cytoplasmic and membranous staining together (total) and membranous staining alone (membranous) were scored separately for HLA class I heavy

chain and $\beta 2m$. As HLA class I complexes are denatured during fixation in formalin, we scored total and membranous staining separately to distinguish free $\beta 2m$ molecules and free heavy chains from those that are assembled as a complex at the cytoplasmic membrane surface.

Statistical analysis

The Wilcoxon rank-sum test was used to identify differences in immunohistochemical staining scores between tumor epithelium and normal layers (surface and deep) of the urothelium for individual APM components. Clinicopathologic information including tumor histology (three groups: urothelial, squamous and mixed/other), UC grade (three groups: 1–2, 3 and 4), pathologic stage (two groups: low, including T1–2, N0, M0 and high, including T3–4 or N1 or M1), and survival were examined with respect to potential associations with immunohistochemical staining score. Wilcoxon rank-sum and Kruskal–Wallis tests were used for univariate two- and three-group comparisons, respectively, for histology, grade and stage. Individual Cox proportional hazards models were fit for survival data using two approaches: (1) including all cancers and adjusting for age, stage, histology, grade, and use of neoadjuvant or adjuvant therapy, and (2) including UC only and adjusting for age, stage, grade and use of neoadjuvant or adjuvant therapy. Hazard ratios estimates, 95% confidence intervals and *P* values were obtained from these models. All tests were performed with $\alpha = 0.05$.

Results

Patient profiles

The 167 patients from whom the cystectomy specimens were obtained included 119 men and 48 women with a mean age of 67 years (range 31–87). One hundred and twenty-eight patients had undergone pelvic lymph node dissection. Of the 19 patients who had been treated with preoperative therapy, 6 had been treated with radiation therapy, 8 with chemotherapy, and 5 with both. The remaining 140 patients received no neoadjuvant therapy. The radiation/chemotherapy status was not known for 8 patients. One hundred and twenty-eight lesions were UC, 24 were squamous cell carcinomas, 7 were adenocarcinomas, 3 were small cell (high-grade neuroendocrine) carcinomas, 2 were mixed urothelial and squamous carcinomas, 2 were sarcomatoid carcinomas, and one was an adenocarcinoma with sarcomatoid foci. Fifty-four tumors were low stage and 113 were high stage. Staging information was incomplete for four patients. Survival data were available for 125 patients with UC; 27 tumors were grade 1 or 2, 81 were grade 3, and 17 were grade 4. The average follow-up period was 3.6 years (range <1 month–18.1 years).

Normal urothelium

Although not assessed statistically because the sample size was too small, comparison of the surface or umbrella layer of normal urothelium with the deeper layers on six normal samples, demonstrated that the average raw scores for staining for $\beta 2m$ (both cytoplasmic and membranous together and membranous alone), calreticulin, HLA class I heavy chain (both cytoplasmic and membranous together and membranous alone), tapasin, TAP1, TAP2 and ERp57 were higher in the surface than in the deeper layers of the urothelium. Delta, MB1, *Z*, LMP2, LMP7 and LMP10 had equal positivity in both levels of the urothelium. Three of the APM components were notable for high staining scores in both types of epithelium, namely delta, *Z* and LMP7, while calreticulin had a high score in the superficial urothelium only. None of the APM proteins had clearly higher staining in the deeper than in the superficial layer of the urothelium (Table 1).

Urothelial carcinoma

Urothelial carcinomas had significantly lower staining scores for membranous $\beta 2m$, calreticulin, delta, Z, LMP7, LMP10, TAP1 and ERp57 than normal superficial urothelium (see Table 1 for *P* values; Fig. 1). UC had significantly lower protein expression of calnexin, delta, Z, LMP7 and LMP10 than the deeper urothelial layer. Interestingly, the staining scores for total $\beta 2m$, both total and membranous HLA class I and tapasin were significantly increased in UC compared to deeper urothelium.

Grade, histologic type and stage

The protein expression level of APM components was not associated with the grade of UC, but was variable in BC of different histologic types. Specifically, the scores were highest in squamous cell carcinomas, intermediate in UC and lowest in tumors of other histologic types (Table 2). These differences reached significance for $\beta 2m$ (membranous only), Z, MICA, LMP2, LMP10, tapasin, TAP1 and TAP2. Furthermore, the staining scores for calnexin, LMP2, LMP7 and LMP10 were associated with stage, being significantly lower in high-stage carcinomas than in low-stage carcinomas (Table 2).

Survival

The protein expression levels of delta (*P* = 0.038) in UC and of calreticulin (*P* = 0.028) in all tumor types, were significantly associated with survival (Fig. 2). Higher staining scores for delta and lower staining scores for calreticulin were associated with lower survival, following statistical adjustments for the histopathologic tumor type if applicable, age and stage, as well as preoperative therapy.

Discussion

The ability to comprehensively study the profile of APM components in formalin-fixed paraffin-embedded tissue has only recently become available, with the majority of previous studies examining the three immunoproteasome components LMP2, LMP7 and LMP10, the TAP subunits TAP1 and TAP2, and tapasin [10, 15–22]. Very little has been previously described regarding the level of APM components in any normal tissues as well as in BC [17, 18, 21]. The present study describes for the first time a comprehensive analysis of APM proteins in normal urothelium and in a large number of BC. The results indicate that, as in other types of solid tumors, defects in APM protein expression are frequent in BC and that some of these defects may have clinical significance [10, 15–18, 22, 23]. As for most carcinomas, very little is known about the mechanisms that underlie abnormal HLA class I antigen expression. Abnormalities may be structural or regulatory, with the latter being more likely for most of the defects in APM protein levels [5].

We found that a high level of protein expression of one of the proteasome components, delta, was independently associated with reduced survival in patients with UC. A reduced level of protein expression of calreticulin, a chaperone protein, was independently associated with a worse outcome for patients with any type of BC. The correlation of delta and calreticulin with outcomes in BC may be due to chance, or may reflect altered immunogenicity of tumor antigens because of defects in the level of these APM components. This possibility is supported by the described role of calreticulin in the immunogenicity of tumor antigens. Calreticulin with associated peptides and not calreticulin alone has been shown to elicit specific immunity to neoplasms [24]. Calreticulin was reduced in urothelial tumors compared to normal superficial epithelium, but not to the normal deep urothelium; this finding may reflect the lower degree of differentiation of the deep urothelium than of the superficial urothelial compartment. As far as we are aware, this

is the first report of prognostic implications for calreticulin or delta in any type of carcinoma.

Various APM proteins have been examined previously in several types of malignancy. For example, primary head and neck squamous cell carcinomas and cervical squamous carcinomas have previously demonstrated low protein levels of calnexin, as have metastatic malignant melanomas [10, 15, 23, 25]. Down-regulation of delta or Z expression has not been previously reported in any carcinoma to our knowledge. In contrast, low expression of LMP7 and LMP10 has been reported in malignant melanoma, and carcinomas of breast, colorectum, cervix and kidney [15–18, 20, 22, 23, 25, 26]. Very few studies of some of the APM components in malignancy have included examination of the corresponding normal tissue; one reported on breast epithelium with antibodies against a few of the APM components, while another examined only LMP2 and LMP7 in normal renal tubular epithelium [17, 18]. Conclusions about the downregulation of APM components in malignant lesions must therefore be interpreted with caution because of the scant information about the level of these components in the normal counterparts. We found that histologically normal urothelium stained for all the APM components tested. Protein expression of LMP molecules would not be expected in normal urothelium; however, as LMP molecules are induced by gamma interferon, the finding of LMP components in normal urothelium may reflect either induction by local interferon or constitutive expression. Higher scores of APM components were typically observed in the superficial compared with the deeper layers.

In analyzing the subunits of the constitutive proteasome and those of the immunoproteasome in normal and malignant urothelium, both superficial and deep urothelium expressed delta, Z, and LMP7 at high levels, with significant decreases in these subunits in UC. Furthermore, LMP10 was also reduced in the carcinomas. Hence, there was a substantial loss of both constitutive proteasome and immunoproteasome subunits in malignant bladder tissue. Basal expression of immunoproteasome subunits has previously been demonstrated in various tissues with normal histology, with the implications not currently understood [27]. In addition, LMP2 and LMP7 proteins have been found at different levels in a variety of carcinomas [10, 16–18, 22, 26, 28]. The molecular mechanisms underlying delta expression or any of the other immune proteasome subunits, is not currently understood in any type of carcinoma.

On comparison with normal superficial urothelium, UC in our study had significantly lower staining scores for eight of the APM components, whereas comparison with normal deeper urothelium revealed a decrease in score for five of the proteins in the cancers. The high staining scores for the three other components, HLA Class 1, β 2m and tapasin, in the cases of UC on comparison to those for the deeper urothelium, contrasts with the decrease in mRNA as reported by Romero et al. [21]. The different results obtained by Romero et al. and ourselves may reflect the different patient populations investigated in the two studies, and/or the lack of correlation between level of mRNA and corresponding protein in malignant cells. This possibility invites one to exercise caution in utilizing level of mRNA as a measure of the corresponding protein in malignant cells.

The lack of significant association of APM protein levels between grades of UC in our study is interesting, given that on multivariate analysis grade appears to have prognostic significance only in superficial BC [29, 30]. Significantly lower staining scores for calnexin, a chaperone protein, and the immunoproteasome components, LMP2, LMP7 and LMP10, were found in high-stage carcinomas when compared with low-stage tumors. This finding may have implications for the mechanisms of immune escape by aggressive tumors. Significant differences were found for seven APM components and MICA between the

different types of bladder cancer, clearly indicating that mechanisms of immune modulation vary with histology.

The current standard therapy for superficial tumors, intravesical bacillus Calmette–Guerin (BCG), has been shown to restore normal MHC class I expression, although its mechanism of action is still not well understood [31]. Other immunotherapeutic agents tested in BC have included interleukins-2 and -12, TNF- α , and interferons- γ and - $\alpha 2\beta$ [32–37]. Interferon- $\alpha 2\beta$ has been used in an effort to increase the T-lymphocytic response in BCG therapy. None of these has proved more efficacious than current therapies, however, and the use of agents such as TNF- α is limited by toxicity. The lengthy list of other immunotherapeutic agents studied, including Keyhole limpet hemocyanin, Bropiramine, Rubratin (*Nocardia rubra*), transforming growth factor-alpha-pseudomonas exotoxin-40 and mistletoe lectin, is testimony to the need to better understand the mechanisms of escape from immune tolerance.

Our study demonstrates that BC has numerous defects in the protein expression of APM components, which likely contribute to their escape from control by the innate immune system. The findings of our study provide data that are important for the design of novel and potentially effective immunotherapeutic approaches for bladder cancer. These may involve manipulations to correct the protein expression of dysregulated APM components.

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Fig. 1.
a Single tissue microarray core of a case of urothelial carcinoma (H&E $\times 100$), **b** immunohistochemical staining of the same tumor for delta, and **c** calreticulin

Fig. 2. Kaplan–Meier survival curves for staining scores for delta in pure urothelial carcinoma, and for calreticulin in all types of bladder carcinoma, with different curves for low- and high-stage carcinoma

Table 1
Differences between average APM scores for the layers of normal urothelium and urothelial carcinoma

APM component	Urothelial carcinoma		Superficial urothelium		P value	Deeper urothelium		P value
	Mean	SD	Mean	SD		Mean	SD	
$\beta 2$ Microglobulin (membranous)	2.3	1.7	3.8	1.2	0.014	1.7	0.5	0.56
$\beta 2$ Microglobulin (total)	3.4	1.6	3.8	1.2	0.23	1.7	0.5	0.002
Calnexin	2.3	1.5	3.0	1.9	0.33	3.6	1.3	0.022
Calreticulin	4.4	1.8	6.0	0.0	0.033	3.5	1.2	0.18
Delta	4.4	1.6	6.0	0.0	0.043	6.0	0.0	0.043
MB1	3.7	2.0	4.5	1.7	0.37	4.5	1.7	0.37
Z	2.4	1.1	6.0	0.0	<0.001	6.0	0.0	<0.001
LMP2	3.5	1.4	4.5	1.6	0.11	4.5	1.6	0.11
LMP7	3.8	1.6	6.0	0.0	0.006	6.0	0.0	0.006
LMP10	2.3	1.5	3.5	1.2	0.014	3.5	1.2	0.014
HLA class I (membranous)	2.7	2.1	2.8	1.2	0.57	0.7	0.8	0.013
HLA class I (total)	2.8	2.0	2.8	1.2	0.61	0.7	0.8	0.004
Tapasin	1.9	1.4	2.8	1.3	0.10	0.6	0.5	0.021
TAP 2	3.2	1.4	3.8	1.2	0.11	2.5	0.5	0.21
TAP 1	1.5	1.4	2.6	0.5	0.015	0.8	1.1	0.22
ERp57	0.5	0.8	1.2	0.8	0.018	0.3	0.5	0.67

Table 2

APM component scores for different histologic types and stages of bladder carcinoma

APM component	Urothelial CA ^b	Squamous cell CA ^c	Other bladder cancer ^d	P value	Low stage	High stage	P value
Total β 2m	3.4	3.7	3.4	0.5915	3.6	3.3	0.3865
Mbr ^a β 2m	2.3	3.4	1.9	0.0205	2.7	2.3	0.3865
Calnexin	2.3	1.9	2.3	0.2653	2.7	2.0	0.0105
Calreticulin	4.4	4.0	3.7	0.2180	4.6	4.2	0.1059
Delta	4.4	4.4	3.7	0.4213	4.7	4.1	0.0580
MB1	3.7	4.0	3.6	0.6937	3.9	3.7	0.5510
Z	2.4	3.1	1.9	0.0049	2.6	2.4	0.0542
LMP2	3.5	4.4	2.9	0.0007	3.9	3.4	0.0468
LMP7	3.8	4.6	3.5	0.0734	4.3	3.7	0.0355
MICA	1.6	1.9	0.9	0.0096	1.8	1.5	0.1195
MICA	2.4	3.1	1.9	0.0018	2.6	2.4	0.0870
LMP10	2.3	2.9	1.1	<0.0001	2.7	2.0	0.0094
Tapasin	1.9	2.3	1.4	0.0414	2.1	1.8	0.2535
TAP2	3.2	4.0	2.6	0.0022	3.5	3.1	0.1979
TAP1	1.5	2.2	1.0	0.0049	1.8	1.5	0.1216
ERp57	0.5	0.6	0.6	0.7013	0.6	0.5	0.1954
Mbr HLA class I	2.7	3.3	2.3	0.2688	2.8	2.7	0.6290
Total HLA class I	2.8	3.3	2.0	0.1443	3.1	2.7	0.2600

^aMembranous^b $n = 117-126$ ^c $n = 22-23$ ^d $n = 13-14$